Pestic. Sci. 1996, **48**, 239–246

Resistance to Sterol Demethylation Inhibitors in *Ustilago maydis*. III. Cross-Resistance Patterns and Sterol Analyses*§

Heilke Wellmann,[a]‡ Karl Schauz[a] & Ralf Tiemann[b]

[a] Department of Biology, NW2, University of Bremen, PO Box 330440, D-28334 Bremen, Germany
[b] Bayer AG, Agrochemicals Division, Chemical Research, Pflanzenschutzzentrum Monheim, D-51368 Leverkusen, Bayerwerk, Germany

(Received 2 August 1995; revised version received 24 January 1996; accepted 14 June 1996)

Abstract: Two spontaneous triadimefon-resistant mutants of *Ustilago maydis*, 151ar/1 and 151ar/3, were investigated with regard to their extent of cross-resistance and their sterol composition to elicit indications about the specificity of the present resistance mechanisms. Testing resistance to various sterol biosynthesis inhibitors and toxicants with different modes of action, it could be demonstrated that, in the mutant 151ar/1, cross-resistance was limited to the sterol demethylation inhibitors (DMIs), whereas, in strain 151ar/3, resistance included most sterol biosynthesis inhibitors studied (DMIs, morpholines, piperidines, allylamines) as well as the unrelated compounds vinclozolin and cycloheximide. Sterol analyses showed that both mutants contained ergosterol as the main sterol component. In comparison with the sensitive reference strain, the mutant 151ar/1 had a slightly elevated content of C-14 methyl sterols, whereas in strain 151ar/3 the amount of ergosterol was increased. Triadimefon caused an accumulation of C-14 methyl sterols and a decrease in ergosterol content in the sensitive strain and the mutant 151ar/1, whereas the other strain 151ar/3 remained unaffected. The results indicate that several resistance mechanisms are probably operating in the two mutants.

Key words: cross-resistance, sterols, DMI-resistance, *Ustilago maydis*, laboratory mutants, triadimefon

1 INTRODUCTION

Fungal sterol biosynthesis has emerged as the currently most important target for antifungal agents. Among the sterol demethylation inhibitors (DMIs) the class of azoles has been developed both for the control of fungal diseases of plants and the treatment of human and animal mycoses. Meanwhile, intensive use of DMIs has led to widespread resistance in several important plant pathogens, e.g. in *Erysiphe graminis* (DC.) Merat f. sp. *hordei* Em. Marchal, *E. graminis* f. sp. *tritici*, *Sphaerotheca fuliginea* (Schlecht ex Fr.) Poll., *Rhynchosporium secalis* (Oudem) J. J. Davis, *Penicillium digitatum* (Pers. ex Fr.) Sacc.[1] Extensive investigations with DMI-resistant laboratory mutants have provided much information about the resistance mechanisms in question. On the other hand, there is hardly any positive information available about the biochemical basis for DMI resistance in field isolates. The understanding of the molecular mechanisms occurring in practice of fungal resistance to this important group of fungicides and antimycotic drugs would be of great value for the prediction of resistance risks and for the design of rational countermeasures and management strategies. Resistance mechanisms found in laboratory mutants are correlated in most cases with a reduced overall fitness, which makes it unlikely that these strains will survive and compete under natural conditions. In the laboratory,

* Presented at the 49th German Plant Protection Conference in Heidelberg on 26–29 September 1994, organised by the Biologische Bundesanstalt für Land- und Forstwirtschaft.
§ Part II. Wellmann, H. & Schauz, K. *Pestic. Biochem. Physiol.* **46** (1993) 55–64.
‡ To whom correspondence should be addressed.

Pestic. Sci. 0031-613X/96/$09.00

fungal mutants are simply selected for fungicide resistance, not for unimpaired fitness. However, any mutants occurring in field populations will have been naturally selected for both fitness and fungicide resistance. As a consequence, resistance genes carrying a fitness penalty will tend to remain rare in natural populations. Therefore, resistance mechanisms which are correlated with a reduced competitive ability should be excluded as a basis for DMI resistance in field isolates. To elucidate the mechanisms of field resistance, only fungal mutants with a quite normal fitness can be useful.

In our studies on the mechanisms of DMI resistance we used spontaneous triadimefon-resistant mutants of *Ustilago maydis* (DC.) Cda. with a quite normal fitness relative to growth rate, sporidial morphology and virulence.[2] The mutants were selected by means of successive adaptation in triadimefon-containing liquid medium, allowing the selection of various spontaneous mutants under continuous fungicide stress over a longer period. The mutants were in long-term competition with each other, leading to an optimum adaptation to the present conditions. Such mutants of high fitness are considered to be suitable model systems for investigations concerning the mechanisms of DMI resistance.

The results of previous studies with protoplasts and of triadimefon uptake indicated that, in the mutant *U. maydis* strain 151ar/1, structural and/or biochemical modifications in the cell wall region might form a penetration barrier to the fungicide and, simultaneously, cause an increased binding of the compound within this area.[3] Thus, a physical exclusion of the effective substance from the target may be working as one resistance mechanism in the mutant strain 151ar/1.

The present paper deals with investigations of cross-resistance patterns and sterol analyses of two DMI-resistant *U. maydis* mutants. Resistance to various sterol biosynthesis inhibitors (SBIs) and to compounds with different modes of action was tested to elucidate the specificity of the resistance mechanisms in question. Sterol analyses were undertaken to check whether a modified sterol pattern might act as a further resistance mechanism in our *U. maydis* mutants.

2 MATERIALS AND METHODS

2.1 Fungal strains

One sensitive and two triadimefon-resistant strains of *U. maydis* were used in this study. The sensitive auxotrophic strain (151s) was a generous gift from Dr Taylor (National Institute for Medical Research, London, UK). The resistant strains (151ar/1, 151ar/3) were derived from the sensitive strain through successive adaptation of sporidia in triadimefon-amended liquid cultures. The strains and their culture conditions have been described in detail in a previous paper.[2] Stock cultures were maintained in anhydrous silica gel[4] under fungicide-free conditions, allowing the dehydrated sporidia to retain their viability over several years. The mutants, which were not completely stable in the absence of triadimefon, had lost some of their original resistance in course of time.

2.2 Fungicides

The following fungicides were used: triadimefon, triadimenol, tebuconazole, flusilazole (triazoles); fenpropimorph, tridemorph (morpholines); fenpropidin (piperidine); terbinafine, naftifine (allylamines); benomyl (benzimidazole); carboxin (carboxamide); vinclozolin (dicarboximide) and cycloheximide (antibiotic). Terbinafine and naftifine were kindly provided by Sandoz Research Institute GmbH (Vienna, Austria), fenpropidin by Ciba-Geigy AG (Dielsdorf, Switzerland) and flusilazole by Du Pont de Nemours GmbH (Münster, Germany). Cycloheximide was purchased from Serva (Heidelberg, Germany) and the other fungicides from Riedel de Haën (Seelze, Germany). With the exception of flusilazole, all fungicides were of analytical grade. Flusilazole was used as the commercial 250 g litre^{-1} EC, 'Captain'. Stock solutions of the fungicides (1–5 mg ml^{-1}) were made in acetone (benomyl, vinclozolin) or ethanol (all other compounds), and diluted with sterile distilled water.

2.3 Toxicity assays

Resistance of the mutant *U. maydis* strains to the various fungicides was determined on agar plates. Fungicides were added to Holliday's simplified complete medium (CM) agar[5] after autoclaving. Sporidia from exponential growth phase were plated on CM agar amended with different fungicide concentrations (100–150 cells per plate, two replicates of two plates per strain and fungicide concentration, respectively) and incubated at 30°C in the dark for six days. Using inhibition of colony diameter as parameter for toxicity, EC_{50} and MIC values (concentrations which inhibited colony diameter by 50% and 100%, respectively) were derived from dosage–response curves. These curves were obtained by testing the various fungicides at eight to twelve different concentrations, comprising the whole range of growth inhibition of each strain.

2.4 Growth conditions for sterol analysis

Exponentially growing sporidia (inoculum density: 2×10^6 cells ml^{-1}) were cultured in large Erlenmeyer flasks (2000 ml) containing 500 ml of CM amended with different triadimefon concentrations (0, 1, 2·5 and 5 μg ml^{-1} for the sensitive strain 151s; 0, 2·5, 5 and 10 μg ml^{-1} for the mutants 151ar/1 and 151ar/3) on a rotary shaker (160 rounds per min^{-1}) at 30°C. In such

large cultures the growth rate of sporidia was reduced because of a more unfavourable oxygen supply, resulting in a later achievement of the stationary phase. After 16–18 h incubation (when the cells were at the end of the log-phase), sporidia were harvested by short centrifugation (5 min at 4000 *g*), washed with distilled water, frozen at − 80°C, and subsequently freeze-dried.

2.5 Sterol extraction

Cholesterol (1 mg in 50 μl methanol) was added as internal standard to freeze-dried sporidia (100 mg) and the sample homogenized in chloroform + methanol (2 + 1 by volume; 80 ml) (Ultra-Turrax). After 16 h extraction, the sample was dried in a vacuum evaporator and then saponified in potassium hydroxide (1 M in ethanol + water, 3 + 2 by volume; 30 ml) for further 16 h. Sterols were extracted with toluene (80 ml), dried in a vacuum evaporator, and taken up in toluene (3 ml). The toluene probe was extracted with a SEP-PAK® silica cartridge (Waters), the cartridge washed with toluene (2 ml), and the sterols were eluted with ethanol (1 ml).

2.6 GLC-MS-analysis of sterols

Sterols were analyzed by GLC using split-injection (1 : 3) on a HP-1 fused silica capillary column with 100% dimethylpolysiloxane (gum) (12 m × 0·2 mm), helium as carrier gas, and a flame-ionization detector or a HP 5970A mass selective detector, respectively. After sample injection (2 μl; injector maintained at 250°C) the oven temperature was held at 200°C for 2 min and then increased to 330°C at 4°C min^{-1}. The cholesterol internal standard was used to calculate the total amount of sterols.

3 RESULTS

3.1 Cross-resistance

The aim of our cross-resistance studies was to determine the extent of cross-resistance in the two *U. maydis* mutants, 151ar/1 and 151ar/3, to elucidate the specificity of the present resistance mechanisms. In a former study it had been shown that both mutants were cross-resistant to the pyrimidine derivatives fenarimol and nuarimol and to the imidazole derivative imazalil.[2] The present investigations used fungicides that interfere with sterol biosynthesis at different target sites as well as compounds with other modes of action.

The results of our toxicity assays are presented in Table 1. The mutant strain 151ar/1 shows cross-resistance to the four triazoles (triadimefon, triadimenol, tebuconazole, flusilazole) and a somewhat reduced sensitivity to cycloheximide, but is sensitive to fenpropimorph, tridemorph, fenpropidin, terbinafine, naftifine benomyl, vinclozolin and carboxin. In contrast, the mutant strain 151ar/3 shows resistance to triazoles, fenpropimorph, tridemorph, fenpropidin, terbinafine, vinclozolin and cycloheximide, but is sensitive to naftifine, benomyl and carboxin.

TABLE 1

Sensitivity of Triadimefon-Resistant *Ustilago maydis* Mutants (151ar/1, 151ar/3) to Various Sterol Biosynthesis Inhibitors and Fungicides with Different Modes of Action

	151s[a]		*151ar/1*				*151ar/3*			
Compound	EC_{50}[b]	*MIC*[c]	EC_{50}[d]		*MIC*[d]		EC_{50}[d]		*MIC*[d]	
Triadimefon[e]	4	6	20	(5)	30	(5)	50	(12·5)	125	(20·8)
Triadimenol	4	6	10	(2·5)	20	(3·3)	30	(7·5)	40	(6·7)
Tebuconazole	0·5	5	5	(10)	10	(2)	10	(20)	20	(4)
Flusilazole	0·5	3	3	(6)	6	(2)	8	(16)	15	(5)
Fenpropimorph	0·5	3	0·5	(1)	3	(1)	5	(10)	10	(3·3)
Tridemorph	1·25	2	1·25	(1)	2	(1)	4	(3·2)	10	(5)
Fenpropidin	1	4	1	(1)	4	(1)	15	(15)	20	(5)
Terbinafine	1	3	2	(2)	5	(1·7)	2	(2)	12	(4)
Naftifine	20	35	20	(1)	35	(1)	30	(1·5)	50	(1·4)
Benomyl	1·5	4	1·5	(1)	4	(1)	2·5	(1·7)	4	(1)
Vinclozolin	15	125	12	(0·8)	70	(0·6)	100	(6·7)	200	(1·6)
Carboxin	0·25	1	0·25	(1)	0·75	(0·75)	0·5	(2)	1·25	(1·25)
Cycloheximide	1	4	3	(3)	8	(2)	5	(5)	20	(5)

[a] Sensitive reference strain.
[b] Concentration of compound (μg ml^{-1}) which inhibited colony diameter by 50%.
[c] Minimum inhibitory concentration (μg ml^{-1}) which inhibited colony growth by 100%.
[d] Values in parentheses are resistance factors ($EC_{50\,res}/EC_{50\,sens}$ and MIC_{res}/MIC_{sens}, respectively).
[e] The mutants have lost some of their original triadimefon resistance in course of time (see Ref. 2).

3.2 Sterol analyses

MS data and relative retention times of the extracted main sterols are given in Table 2. The results of the sterol analyses carried out with the three *U. maydis* strains are summarized in Tables 3–5.

Untreated controls of both sensitive and resistant sporidia contain ergosterol as the main sterol component and minor amounts of several ergosterol precursors occurring normally in the biosynthetic pathway. Total sterol content does not differ significantly among the three strains. However, their sterol patterns differ quantitatively in some way from one another. In the sensitive reference strain 151s, the amount of 4,4-dimethyl- and 4α-methylfecosterol is relatively high, making up 25% of total sterols, whereas in the resistant mutants these two sterols are only present in traces. The mutant strain 151ar/3 has a higher ergosterol content (81%) than the sensitive strain (60%). The mutant 151ar/1 is characterized by a comparatively high amount of 24-methylenedihydrolanosterol and an elevated level of obtusifoliol, whereas the sensitive strain 151s and the mutant 151ar/3 contain only traces of obtusifoliol.

TABLE 2
Mass Spectra and Relative Retention Times of Extracted Sterols

		Sterols[a]							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Ion[b]	$[M]^+$, m/z	*396*	*440*	*426*	*426*	*412*	*398*	*400*	*412*
		(Rel. int., %)							
$[M]^+$		50	14	31	49	49	26	76	31
$[M\text{-}CH_3]^+$		3	33	95	25	29	3	24	86
$[M\text{-}H_2O]^+$		2	2	—	7	4	3	—	—
$[M\text{-}CH_3\text{-}H_2O]^+$		87	15	13	12	16	48	5	10
$[M\text{-SC}]^+$		11	—	18	31	40	2	28	6
$[M\text{-SC-}42]^+$		8	7	11	14	21	21	28	30
$[M\text{-SC-}H_2O]^+$		52	—	10	13	5	—	83	4
$[M\text{-SC-}42\text{-}H_2O]^+$		40	10	5	4	14	19	43	17
$[M\text{-SC-}CH_3\text{-}C_3H_5]^+$		—	33	59	18	13	—	—	62
RRT[c]		1·063	1·213	1·136	1·224	1·141	1·110	1·121	1·090

[a] Sterol identification: 1 = ergosterol (Δ5,7,22-ergostatrienol); 2 = 24-methylenedihydrolanosterol (4,4,14α-trimethyl-Δ8,24(28)-ergostadienol); 3 = obtusifoliol (4α, 14α-dimethyl-Δ8,24(28)-ergostadienol); 4 = 4,4-dimethylfecosterol (4,4-dimethyl-Δ8,24(28)-ergostadienol); 5 = 4α-methylfecosterol (4α-methyl-Δ8,24(28)-ergostadienol); 6 = Δ5,7-ergostadienol; 7 = Δ7-ergostenol; 8 = 14α-methylfecosterol (14α-methyl-Δ8,24(28)-ergostadienol).

[b] SC = Sterol side-chain.

[c] Relative retention time (cholesterol = 1·000).

TABLE 3
Sterol Composition and Total Sterol Content of the Sensitive *Ustilago maydis* Strain 151s after Treatment with Different Triadimefon Concentrations

	Composition (%) (±SD)[a]			
		Triadimefon treatment		
Sterols	*Untreated control*	*1 μg ml⁻¹*	*2·5 μg ml⁻¹*	*5 μg ml⁻¹*
Ergosterol	59·8 (±5·1)	37·2 (±6·1)	31·1 (±8·7)	16·6 (±5·3)
24-Methylenedihydrolanosterol	5·5 (±1·3)	13·7 (±1·4)	32·9 (±5·7)	64·2 (±0·8)
Obtusifoliol	tr.[b]	6·0 (±1·0)	9·5 (±0·1)	15·6 (±1·3)
4,4-Dimethylfecosterol	15·7 (±2·5)	12·3 (±2·3)	5·8 (±2·0)	tr.
4α-Methylfecosterol	9·6 (±1·2)	8·9 (±7·7)	2·7 (±3·9)	tr.
Δ5,7 Ergostadienol	6·9 (±2·4)	12·8 (±11·7)	12·9 (±4·4)	1·5 (±2·1)
Δ7 Ergostenol	tr.	3·7 (±3·3)	4·9 (±1·6)	1·9 (±2·8)
Unidentified sterols	2·4 (±2·1)	5·5 (±9·5)	tr.	tr.
Sterol content (μg mg^{-1} dry weight)	3·59 (±0·84)	3·39 (±0·38)	3·47 (±0·28)	4·38 (±1·48)

[a] n = 3–4.

[b] Traces (below the quantifiable level).

TABLE 4
Sterol Composition and Total Sterol Content of the Mutant *Ustilago maydis* Strain 151ar/1 after Treatment with Different Triadimefon Concentrations

Sterols	*Composition (%) (± SD)*[a]			
	Untreated control	*Triadimefon treatment*		
		2·5 μg ml^{-1}	*5 μg ml*$^{-1}$	*10 μg ml*$^{-1}$
Ergosterol	50·5 (±3·9)	51·9 (±3·5)	47·8 (±5·8)	19·3 (±2·1)
24-Methylenedihydrolanosterol	33·1 (±4·4)	29·7 (±2·6)	27·4 (±4·6)	48·8 (±3·5)
Obtusifoliol	8·8 (±1·1)	9·5 (±1·2)	13·4 (±2·5)	20·7 (±2·6)
14α-Methylfecosterol	2·3 (±2·5)	3·0 (±2·5)	6·0 (±4·8)	6·0 (±6·0)
4,4-Dimethylfecosterol	tr.[b]	tr.	tr.	tr.
4α-Methylfecosterol	tr.	tr.	tr.	tr.
Δ5,7 Ergostadienol	3·4 (±3·1)	3·6 (±3·1)	2·8 (±3·3)	tr.
Δ7 Ergostenol	2·0 (±2·0)	2·3 (±1·7)	2·5 (±3·0)	5·2 (±1·6)
Sterol content (μg mg^{-1} dry weight)	4·38 (±0·43)	4·15 (±0·42)	3·77 (±0·66)	2·45 (±0·42)

[a] $n = 4$–5.
[b] Traces (below the quantifiable level).

Treatment of sensitive sporidia with increasing triadimefon concentrations causes decreasing amounts of ergosterol and an accumulation of C-14 methyl sterols (24-methylenedihydrolanosterol and obtusifoliol). This typical 'azole effect' is also associated with a decrease of 4,4-dimethyl- and 4α-methylfecosterol. Total sterol content remains unchanged (Table 3). Triadimefon concentrations resulting in a strong inhibition of ergosterol biosynthesis in the sensitive strain 151s do not cause any alterations in the sterol profiles of the resistant mutants. Increasing the triadimefon concentration to 10 μg ml^{-1}, an 'azole effect' together with a decrease in total sterol content can be observed in the mutant strain 151ar/1. However, the low amount of 14α-methylfecosterol remains unaffected (Table 4). With the other mutant, 151ar/3, a slightly reduced ergosterol content and a somewhat increased level of 24-methylenedihydrolanosterol is seen at the highest fungicide concentration tested, but an unequivocal 'azole effect' cannot be recognized. Total sterol content does not change significantly (Table 5).

4 DISCUSSION

Two spontaneous triadimefon-resistant mutants of *U. maydis*, 151ar/1 and 151ar/3, were investigated with regard to their extent of cross-resistance and their sterol composition. The mutants differed in their patterns of cross-resistance. While strain 151ar/1 was resistant only

TABLE 5
Sterol Composition and Total Sterol Content of the Mutant *Ustilago maydis* Strain 151ar/3 after Treatment with Different Triadimefon Concentrations

Sterols	*Composition (%) (± SD)*[a]			
	Untreated control	*Triadimefon treatment*		
		2·5 μg ml^{-1}	*5 μg ml*$^{-1}$	*10 μg ml*$^{-1}$
Ergosterol	81·1 (±3·0)	78·9 (±4·1)	76·5 (±7·5)	67·1 (±10·0)
24-Methylenedihydrolanosterol	12·6 (±3·1)	11·4 (±1·2)	12·1 (±0·9)	17·8 (±2·0)
Obtusifoliol	0·4 (±0·9)	0·9 (±1·1)	0·5 (±1·0)	tr.[b]
14α-Methylfecosterol	tr.	tr.	tr.	tr.
4,4-Dimethylfecosterol	1·7 (±2·3)	2·4 (±2·9)	3·9 (±4·9)	5·8 (±7·0)
4α-Methylfecosterol	1·3 (±1·8)	1·5 (±1·8)	2·5 (±2·9)	4·0 (±4·6)
Δ5,7 Ergostadienol	0·2 (±0·5)	tr.	tr.	tr.
Δ7 Ergostenol	2·7 (±1·7)	4·7 (±1·0)	4·4 (±1·7)	5·2 (±1·6)
Sterol content (μg mg^{-1} dry weight)	3·83 (±0·79)	3·83 (±0·48)	3·35 (±0·24)	2·84 (±0·50)

[a] $n = 4$–5.
[b] Traces (below the quantifiable level).

to DMIs, i.e. to inhibitors of sterol C-14 demethylase, resistance of 151ar/3 included most sterol biosynthesis inhibitors investigated (DMIs, morpholines, a piperidine, allylamines) as well as the unrelated compounds vinclozolin and cycloheximide.

Usually, cross-resistance exists among the DMIs, but is absent to morpholines, piperidines or allylamines,[6–9] sometimes even displaying an increased sensitivity to morpholines,[10] while, *vice versa*, fungal strains resistant to morpholines or allylamines usually are sensitive to DMIs.[11–13] This fact can be referred to the different target sites in the sterol biosynthetic pathway: DMIs inhibit sterol C-14 demethylase, whereas morpholines and piperidines act at the Δ14-reductase and the Δ8 → Δ7-isomerase and allylamines block the squalene epoxidase. The limited cross-resistance pattern shown for our *U. maydis* mutant 151ar/1 implies a specific resistance mechanism possibly acting at the target site of fungicide action.

However, resistance to SBIs can also be unspecific, comprising DMIs, morpholines and allylamines, as has been described for *Ustilago avenae* (Pers.) Rostr.,[14] *U. maydis*,[15] *Monilinia fructicola* (Wint.) Honey,[16] *Pseudocercosporella herpotrichoides* (Fron.) Deighton,[17] and for our mutant *U. maydis* strain 151ar/3. The fact that 151ar/3 shows additional reduced sensitivity to fungicides with quite different modes of action points to an unspecific resistance mechanism, which may be the result of a reduced uptake of inhibitor. In a similar case, multiple cross-resistance in *Saccharomyces cerevisiae* Hansen to eight different inhibitors of respiration or protein synthesis could undoubtedly be related to a reduced uptake of effective substances.[18] In imazalil-resistant mutants of *Aspergillus nidulans* (Eidam) Wint. resistance was based on a multigenic system; mutations at eight different loci led to pleiotropic effects, e.g. resistance to the unrelated compounds cycloheximide, chloramphenicol, neomycin and acriflavin.[19] In some of these cases a reduced uptake of fungicide, caused by a higher energy-dependent efflux activity compared to the wild-type strain, was found to function as a resistance mechanism.[20]

Simultaneous resistance to DMIs and fungicides with other modes of action (benzimidazoles, hydroxypyrimidines, organophosphates, carboxin, dicarboximides) has been found both in field isolates and in laboratory mutants,[21–26] but the reason for this phenomenon has not yet been clarified. In previous studies with the mutant *U. maydis* strain 151ar/3 we could demonstrate that DMI resistance has a polygenic basis,[2] that means, different single gene mutations ('minor genes') causing only small effects on resistance had accumulated in a single cell, where they interacted positively to produce higher degrees of resistance.[27] It seems reasonable to assume that the different gene mutations represent several more or less specific resistance mechanisms, which as a whole confer resistance on the cell not only to SBIs but also to unrelated compounds.

In the second part of our studies, sterol analyses were carried out to examine whether alterations in the sterol biosynthetic pathway might contribute to DMI resistance in the *U. maydis* mutants. In neither mutant was any major change in sterol patterns found, which would point to a block in sterol biosynthesis. Ergosterol is the predominant sterol component in all three strains. In contrast, ergosterol is absent in fungal mutants with a defect in C-14 demethylation and/or C5–6 desaturase. Such mutants accumulate abnormal C-14 methyl sterols and show a greatly impaired overall fitness compared with the wild-type. They have been described for *Ustilago*[14,28,29] and yeasts,[30–32] but, until now, no similar field resistant mutants of plant pathogenic fungi have been identified that lack C-14 demethylase activity.

Both mutants differed in their quantitative sterol composition from the sensitive reference strain 151s, pointing to slight modifications in the metabolism rates. The mutant strain 151ar/1 contained a comparatively high amount of 24-methylenedihydrolanosterol and an elevated level of obtusifoliol. This accumulation of C-14 methyl sterols may be indicative of a reduction in C-14 demethylase activity, which, however, does not affect fungal growth. Triadimefon concentrations that led to a strong inhibition of ergosterol biosynthesis and sporidial growth in the sensitive strain 151s did not cause any alterations in the sterol pattern or growth rate of the mutant 151ar/1. At higher triadimefon concentrations, however, a typical 'azole effect' was observed, indicating that the sterol C-14 demethylation in this mutant remained sensitive to the DMIs, but at a lower level. A possible explanation of this fact would be a mutation at the cytochrome $P450_{14DM}$ partially affecting the catalytic activity of the enzyme and reducing the affinity to the DMIs. A similar resistance mechanism was assumed for *Candida albicans* (Robin) Berkhout,[33] *Nectria haematococca* Berk. & Br. var. *cucurbitae*,[34] and field isolates of barley powdery mildew and *R. secalis*.[1]

Azole-treated sensitive *U. maydis* sporidia are often found to accumulate higher amounts of 14α-methylfecosterol and sometimes 14α-methylfecosterol-3,6-diol in addition to 24-methylenedihydrolanosterol and obtusifoliol.[35–39] 14α-methylfecosterol is able to support sporidial growth in the more or less complete absence of ergosterol and other desmethyl sterols,[38,39] whereas the 3,6-diol does not. A defective sterol C5–6 desaturase, which resulted in a reduced amount of 14α-methylfecosterol-3,6-diol and an increased proportion of 14α-methylfecosterol in treated cells compared to the parent strain, was responsible for azole resistance in an *U. maydis* mutant.[39] However, in our case, only the mutant strain 151ar/1 presented 14α-methylfecosterol in a low amount that did not alter significantly under azole treatment. The 3,6-diol was not detected. Obviously, C-4 demethylation of obtusifoliol to 14α-

methylfecosterol does not play any role in the azole resistance of our *U. maydis* mutants.

Based on the results of the sterol analyses and the studies of cross-resistance patterns, the following hypothesis for the mutant strain 151ar/1 is made: a modified demethylation rate of the target enzyme cytochrome $P450_{14DM}$ causes a slight accumulation of C-14 methyl sterols, at which ergosterol, however, is still synthesized in sufficient amounts, preventing significant impairment of fungal growth. This modification simultaneously decreases the binding affinity of the enzyme to the DMIs, so that considerably higher fungicide concentrations are necessary to inhibit sterol C-14 demethylation. This hypothetical resistance mechanism would correspond with the limited cross-resistance pattern of strain 151ar/1, which included only the DMIs. However, the additional reduced sensitivity to the unrelated compound cycloheximide implies a further resistance mechanism that might be based on structural and/or biochemical modifications in the cell wall region forming a penetration barrier to the fungicide. This mechanism has been assumed in a previous paper[3] and probably contributes to DMI resistance, too. So, at least two different resistance mechanisms seem to operate in the mutant strain 151ar/1.

The other mutant, 151ar/3, showed a relatively high ergosterol content, which could possibly be explained by an increased efficiency of ergosterol biosynthesis, compensating the inhibitory effect of DMIs. Treatment of sporidia with the highest triadimefon concentration (10 $\mu g\ ml^{-1}$) did not cause any significant changes in the sterol profile of the mutant. An increased efficiency of ergosterol biosynthesis may result from a higher constitutive level of cytochrome $P450_{14DM}$ or induced overexpression of the enzyme. Overproduction of the target enzyme as a basis for resistance has been described for a terbinafine-resistant *U. maydis* mutant[40] and a transformed *S. cerevisiae* strain with resistance to fenpropidin.[41] In a *Candida glabrata* (Anderson) Meyer & Yarrow isolate reduced uptake of inhibitor together with an increased C-14 demethylase activity was involved in azole-resistance.[42] Transformation of sensitive *C. albicans* and *S. cerevisiae* with multiple copies of the *C. albicans* gene coding for cytochrome $P450_{14DM}$ resulted in an overexpression of the enzyme. However, the level of resistance observed was not as high as might have been expected.[43] So, an increase in the quantity of sterol C-14 demethylase appears unlikely to cause dramatic changes in DMI resistance.

The resistance pattern of the mutant 151ar/3 included various SBIs and some other unrelated compounds. Therefore, another quite unspecific resistance mechanism must be operating. A restricted uptake of inhibitor into fungal cells might be a reasonable explanation. But analyses of fungicide content of triadimefon-treated sporidia using a triadimefon-specific ELISA did not show any significant differences between the resistant mutant 151ar/3 and the sensitive reference strain 151s (unpublished results). However, the method used cannot distinguish between the cell-wall-bound and the intracellular portion of the inhibitor, leaving uncertainties with regard to the exact fungicide localization. It may be possible that the cell wall functions as a penetration barrier and, simultaneously, causes an increased binding of inhibitors to this region. Such a mechanism based on structural and/or biochemical changes in the cell wall region has been assumed for the DMI-resistant mutant 151ar/1.[3] Considering the fact that strain 151ar/3 was derived from strain 151ar/1 through further adaptation,[2] one could suppose that further alterations in the cell wall region might have led to a more unspecific penetration barrier resulting in resistance to several unrelated fungicides.

The results of our studies indicate that in both mutants probably more than one resistance mechanism is operating. Whether a reduced affinity of the C-14 demethylase or an increased activity of the target enzyme really contributes to DMI resistance, will be the subject of further investigations.

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